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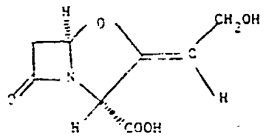
(54) CLAVULANIC ACID PRODUCTION

(71) We, GLAXO LABORATORIES LIMITED a British company of Greenford, Middlesex, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to improvements in or relating to the fermentation of strains of *Streptomyces clavuligerus*.

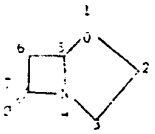
In our British Patent Specification No. 1,543,563 we have described the isolation, from fermentations of *Streptomyces clavuligerus*, of the carboxylic acid having the formula (I) (clavulanic acid).



(I)

and salts thereof in pure form. British Patent Specification No. 1,508,977 also describes the fermentation and isolation of clavulanic acid.

Clavulanic acid may be named with reference to "clavam"; the name given to the parent heterocycle of formula A



(A)

by analogy with the term "cepham" used in the naming of cephalosporin compounds in J. Amer. Chem. Soc., 1962, *84*, 3400. Thus, the compound of formula (I) is named (3R, 5R, Z)-2-(hydroxyethylidene)clavam-3-carboxylic acid.

Clavulanic acid and its salts have been found to exhibit antibacterial activity against a range of gram-positive and gram-negative microorganisms and have further been found to possess the ability to inhibit ß-lactamase enzymes produced by a range of gram-positive and gram-negative microorganisms.

The above British Patent Specification No. 1,508,977 suggests relatively broad pH ranges for fermentation and specifically describes fermentation at pH 7.0 or above. British Patent No. 1,315,177 which describes fermentation of *Streptomyces clavuligerus* for the production of other antibiotics indicates a pH range rising to from 6.7 to 7.5 or above.

We have now found, however, that the amount of clavulanic acid produced in the fermentation of *Streptomyces clavuligerus* may be increased to a surprising degree if the

fermentation is carried out under conditions of strict pH control in the range 6.3 to 6.7.

Accordingly, we provide a process for the fermentation of *Streptomyces clavuligerus* to produce clavulanic acid which comprises cultivating a strain of *Streptomyces clavuligerus* in a nutrient medium therefor wherein the pH of the medium is maintained for the greater part of the fermentation time within the range 6.3 to 6.7. A pH of about 6.5 is optimal.

While substantial benefit accrues from pH control within the stated limits over the greater part of the fermentation time, it is generally preferred that such control should be effected throughout the fermentation time. However, it may not be necessary to control pH within said limits during the initial growth phase, for example during the first thirty hours, although in general, the pH of the medium should not at any time fall outside the range 6-8 for any significant period of time.

We have found the use of the pH range 6.3-6.7 so advantageous that over twice as much clavulanic acid may be produced at, for example, pH 6.5 than is produced at a pH of 6.0 or 7.0. In some experiments that we have performed, the yield of clavulanic acid almost tripled when fermentation was carried out at pH 6.5 as compared with the result at pH 6.0 or 7.0.

We have found it most preferable to control the pH at which fermentation is carried out automatically. This may be effected using a pH-controlling device whereby metered amounts of aqueous mineral or carboxylic acid (e.g. hydrochloric, sulphuric, citric or acetic acid) and/or a base (e.g. gaseous ammonia or aqueous sodium or potassium hydroxide or carbonate or ammonium hydroxide) are added automatically during the fermentation process in response to changes in pH. Aqueous solution of acids or bases used for pH adjustment preferably contain 5% to 15% of said acid or base.

With the exception of the very close control of the pH, the fermentation process for the production of clavulanic acid from *Streptomyces clavuligerus* may be effected by conventional means, i.e. by cultivating the *Streptomyces clavuligerus* in the presence of assimilable sources of carbon, nitrogen and mineral salts. Cultivation will preferably be carried out by submerged culture under aerobic conditions.

Assimilable sources of carbon, nitrogen and minerals may be provided by simple and/or complex nutrients. Sources of carbon will generally include glucose, starch, glycerol, maltose, sucrose, molasses, carboxylic acids, dextrin and/or lactose.

Sources of nitrogen will generally include soyabean meal, corn steep liquors, distillers solubles, yeast extracts, cottonseed meal, peptones, casein and amino acid mixtures. Urea and other amides may also be used.

Nutrient mineral salts which may be incorporated into the culture medium include the generally used salts capable of yielding, for example, sodium, potassium, ammonium, iron, calcium, magnesium, zinc, nickel, cobalt, manganese, phosphate, sulphate, chloride and/or carbonate ions.

An antifoam agent will generally be present to control excessive foaming and may be added at intervals as required.

Cultivation of the *Streptomyces clavuligerus* will generally be effected at a temperature of from 20°-37°C, preferably of from 25°-30°C, and will desirably take place with agitation, e.g. by shaking or else by stirring and aeration. The growth medium may initially be innoculated with a small quantity of sporulated suspension of the microorganism but in order to avoid a growth lag a vegetative inoculum of the organism may be prepared by inoculating a small quantity of culture medium with the spore form of the organism, and the vegative inoculum obtained may be transferred to the fermentation medium, or, more preferably to a seed stage where further growth takes place before transfer to the principal fermentation medium.

The microorganism is a strain of *Streptomyces clavuligerus*. We have found strain NRRL 3585 and selectants and mutants thereof to be particularly satisfactory strains for the production of clavulanic acid. the morphology of the said strain NRRL 3585 is described in the above British Patent No. 1,315,177.

As used herein, the term 'mutant' will include any mutant strain which arises either spontaneously or as a result of the action of an external agent, which may be either deliberately applied or otherwise. Mutant strains may be produced by a variety of methods including those outlined in Techniques for the Development of Micro-Organisms by H. I. Adler in "Radiation and Radioisotopes for Industrial Microorganisms", Proceedings of the Symposium, Vienna, 1973, p. 241, International Atomic Energy Authority. These methods include

i) Ionising radiation, for example X- and γ-rays, uv light, uv light in the presence of a photosensitising agent, for example 8-methoxypsoralen; nitrous oxide; hydroxylamine; pyrimidine base analogues, e.g. 5-bromouracil; acridines; alkylating agents, e.g. ethyl methane-sulphonate or mustard gas; hydrogen peroxide; phenols; formaldehyde; heat: and

ii) genetic techniques, such as recombination, transduction, transformation, lysogenisation, lysogenic conversion and selective techniques for spontaneous mutants.

As used herein, the term 'selectant' means a strain of the microorganism derived from a

colony selected from the parent strain which has been cultivated in such a way as to provide a strain having one or more properties which are qualitatively or quantitatively different from those of the parent strain, e.g. resistance to substances produced in fermentation. Such a selectant may, of course, be a spontaneous mutant of the parent microorganism but in some cases it may not be.

In a preferred embodiment of the fermentation, therefore, a slope of *Streptomyces clavuligerus* NRRL 3585, or a mutant or selectant thereof, may be used to inoculate a medium comprising sources of assimilable carbon, e.g. sucrose or dlycerol, assimilable nitrogen, e.g. tryptones, or complex mixtures of assimilable carbon and nitrogen, e.g. distillers solubles and yeast extracts, and nutrient minerals. This medium may be allowed to grow for up to 3 days at from 25-30°C with agitation.

The developed inoculum thus formed may then be used to inoculate (in a quantity of up to about 10%) a nutrient medium containing similar sources of assimilable carbon, nitrogen and minerals. This fermentation will desirably be carried out batchwise at from 25-30°C for from 3-10 days with agitation and aeration at a pH of 6.5, the pH being controlled by automatic additions of dilute mineral acid and base as described above.

Clavulanic acid formed during the fermentation may be estimated by the cup-plate agar assay system described by Lees and Tootill in Analyst, 1955, *80*, (947), pp. 95-110; pp. 110-123 and in Analyst, 1955, *80* (952), pp. 531-535 against *Acinetobacter sp.*

Isolation of clavulanic acid, particularly in the form of its lithium salt, is described in detail in our above-mentioned German OLS. In general, after fermentation, the clavulanic acid is preferably isolated from the nutrient medium in one or more stages as the lithium salt which, if desired, is converted by ion exchange into clavulanic acid or a salt thereof other than the lithium salt. The fermentation broth may thus be clarified, applied to a charcoal column to adsorb the clavulanic acid and/or its salts which are then eluted with aqueous solvent, e.g. aqueous acetone, and the eluate adsorbed onto a basic ion exchange resin. The resin adsorbate may then be eluted with an aqueous solution of a lithium salt such as lithium chloride and the eluate concentrated to precipitate the lithium clavulanate in a particularly high state of purity.

The invention will now be more particularly described in the following Examples, which should not be considered as limiting the invention.

Example 1

a) *Inoculum development*

Sterile distilled water (10ml) was added to a 14 day old malt/yeast extract agar slope of *Streptomyces clavuligerus* NRRL 3585 and a suspension made. A portion (1.5 ml) of this suspension was used to inoculate 150 ml of a steam-sterilised medium containing:—

	g/litre
sucrose	20
distillers solubles	15
yeast extract	5
K_2HPO_4	0.2
tryptone	5
glycerol	10
water	to 1 litre

in a 2 litre Florence flask.

This flask was incubated at 26°C for 48 h at 220 rev./min on a rotary shaker with a 2 inch throw.

b) *Fermentation*

A number of such developed inocula were used each to inoculate (3.75%) a series of 5 litre fermenters each containing 4 litres of a steam-sterilised medium containing:—

	g/litre
distillers solubles	5.2
casein hydrolysate	5.2
soya bean meal	21
soluble starch	47
glucose	7.8
ferrous sulphate (7 H_2O)	0.1
antifoam (polyglycol) 0.5 ml/litre	
water to 1 litre	

The fermentations were incubated at 28°C with aeration (0.75 vol/vol/min) and agitation (750 rev./min; 2 x 3 inch diameter impellers) for a period of 92 hours. Fermentation pH was controlled as indicated from 32 h after inoculation, using an automatic device whereby ammonia solution (10% v/v of 0.880 ammonia solution) or hydrochloric acid solution (5% v/v concentrated HCl) were added as required. Culture pH was measured by means of a

steam-sterilisable glass electrode (Pye Unicam Ltd., York St, Cambridge). The electrode was coupled to a pH monitor (Model 539, Pye Ether, Caxton Way, Stevenage) and control system which regulated the supply of acid or alkali to the culture vessel by means of Watson Marlow pumps (Watson Marlow Ltd., Falmouth, Cornwall).

The clavulanic acid content of the fermentation medium was estimated on samples taken during the fermentation using a cup-plate agar assay (Lees, K.A. & Tootill, J.P.R., Analyst, 1955, *80* (947), 95-110; ibid 110-123; *80* (952), 531-535) against *Acinetobacter sp.* and the maximum titre of clavulanic acid was determined; it was found that:

pH control to	*μg/ml clavulanic acid (max. titre)*
5.5	40
6.0	187
6.5	561
7.0	218
7.5	97

Example 2

In a second experiment carried out as in Example 1, the fermentations were controlled to pH 6.5 with control starting at various times after inoculation, and the clavulanic acid content was again estimated on samples taken during the fermentation.

pH control to 6.5 from (h)	*μg/ml clavulanic acid (max. titre)*
0	754
20	474
32	478
44	339
56	364

Similar results were obtained starting pH control to 6.5 from 0 hour using acetic acid solution (10% v/v glacial acetic acid) in place of hydrochloric acid solution.

Example 3

A third experiment was carried out under the conditions as in Example 1 except that hydrolysed casein was omitted from the medium, agitation was at 550 rev./min (2x3.5 inch diameter impellers) and the fermentation temperature was 30°C. Fermentation pH was controlled as before to 6.5 from the start of the fermentation but was terminated at various time after inoculation. Clavulanic acid content was estimated on samples taken during the fermentation.

pH control terminated at h	*μg/ml clavulanic acid (max. titre)*
20	160
32	125
44	306
56	289
68	550
92	508

WHAT WE CLAIM IS:—

1. A process for the fermentation of *Streptomyces clavuligerus* to produce clavulanic acid which comprises cultivating a strain of *Streptomyces clavuligerus* in a nutrient medium therefor wherein the pH of the medium is maintained for the greater part of the fermentation time within the range 6.3 to 6.7.
2. A process as claimed in claim 1 in which the pH of the medium is maintained within the range 6.3 to 6.7 throughout the fermentation time.
3. A process as claimed in claim 1 or claim 2 in which the pH of the medium is maintained at about 6.5.
4. A process as claimed in any of claims 1 to 3 in which the pH is controlled automatically.
5. A process as claimed in claim 4 in which metered amounts of aqueous mineral or carboxylic acid and/or a base are added automatically during the fermentation process in response to changes in pH.
6. A process as claimed in any of claims 1 to 5 in which cultivation is carried out by submerged culture under aerobic conditions.
7. A process as claimed in any of the preceding claims in which the nutrient medium comprises assimilable sources of carbon, nitrogen and mineral salts.
8. A process as claimed in claim 7 in which glucose, starch, glycerol, maltose, sucrose, molasses, dextrin and/or lactose is included as a carbon source.
9. A process as claimed in any of claims 1 to 8 in which soyabean meal, corn steep liquors, distillers solubles, yeast extracts, cottonseed meal, peptones, casein, amino acid mixtures, and/or urea and other amides are used as nitrogen source.
10. A process as claimed in any of claims 1 to 9 in which salts capable of yielding sodium,

potassium, ammonium, iron, calcium, magnesium, zinc, nickel, cobalt, manganese, phosphate, sulphate, chloride and/or carbonate ions are used as nutrient mineral salts.

11. A process as claimed in any of claims 1 to 10 in which cultivation is effected at a temperature of from 20° to 37°C.

12. A process as claimed in claim 11 in which cultivation is effected at a temperature of from 25°-30°C.

13. A process as claimed in any of claims 1 to 12 in which the nutrient medium is agitated during fermentation.

14. A process as claimed in any of claims 1 to 13 in which the strain of *Streptomyces clavuligerus* is NRRL 3585 or a mutant thereof.

15. A process as claimed in any of claims 1 to 14 in which after fermentation, clavulanic acid is isolated from the nutrient medium in one or more stages as the lithium salt which, if desired, is converted by ion exchange into clavulanic acid or a salt thereof other than the lithium salt.

16. A process as claimed in claim 1 substantially as herein described.

17. A process as claimed in claim 1 substantially as herein described with reference to any of Examples 1 to 3.

18. Clavulanic acid or a salt thereof whenever prepared using a process as claimed in any of claims 1 to 17.

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